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(54) Title: PREGNANCY INDUCED HYPERTENSION AND ECLAMPSIA IMMUNOASSAY AND REAGENTS

(57) Abstract

Preeclampsia, pregnancy induced hypertension (PIH) and eclampsia are determined by identifying the presence of an A134-binding cell marker in a sample of blood, plasma or serum of a pregnant woman using a sandwich or competition immunoassay. Cellular fibronectin marker in a sample is determined by binding with an anti-(cellular fibronectin) antibody. Reagents for these methods are also an aspect of the invention.

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PREGNANCY INDUCED HYPERTENSION AND ECLAMPSIA IMMUNOASSAY AND REAGENTS

FIELD OF THE INVENTION

This invention relates to methods, reagents and kits for detection of pregnancy induced hypertension (PIH) or preeclampsia during pregnancy. In particular, this invention is directed to the determination of PIH or preeclampsia by testing whole blood, serum or plasma samples for the presence of fibronectin isoforms which are indicative of the presence of PIH or preeclampsia.

BACKGROUND OF THE INVENTION

Preeclampsia, eclampsia and pregnancy induced hypertension (PIH) are characterized by elevated blood pressure, proteinuria, and edema. The cause and nature of these disorders is only partially understood. Preeclampsia and PIH are often used to designate the same disorders. The term "preeclampsia" is used hereinafter, for purposes of clarity of explanation, not by way of limitation, to broadly include preeclampsia, pregnancy induced hypertension, and eclampsia. Although considered to be relatively rare in the United States, preeclampsia occurs worldwide in from 2 to 35 percent of pregnancies, depending on diagnostic criteria and study population. Deaths from preeclampsia are nearly equal to those from eclampsia in a recent report by Redman, C., Brit. Med. J. 296:1209-1210 (April, 1988). However, tests for these conditions are often ambiguous, and diagnosis of these conditions have often not been possible until the condition has progressed. A reliable test for early diagnosis of this condition is critically needed.

A review of the role of prostaglandins in preeclampsia was published by Friedman, S. Obstet. Gynecol. 71:122-137 (1988). Examination of maternal fluids for metabolic markers for PIH and preeclampsia has revealed that urine levels of 2,3-dinor-6-keto PG $F_{1\alpha}$ increase during this condition, Ob/Gyn Topics 2:5

(1987). Levels of other substances in the blood have also been studied.

U.S. Patent 4,840,894 describes a method for diagnosing essential hypertension by detecting an integral membrane with an essential hypertension associated calcium-binding protein.

A number of studies have focused on the general increase in fibronectin levels in blood during these disease processes: Graninger, W. et al, Europ. J. Obstet. Gynec. Reprod. Biol. **19**:223-229 (1985); Hess, L. et al, Obstret. Gynecol. **68**:25-28 (1986); Lazarchick, J. et al, Am. J. Ob. Gyn. **154**:1050-1052 (1986); Ericksen, H. et al, Acta. Obstet. Gynecol. Scand. **66**:25-28 (1987); and Saleh, A. et al, Obstet. Gynecol. **71**:719 (1988), for example. Although fibronectin levels in the blood were reportedly higher with PIH and preeclampsia, the degree of increase varied with each individual and stage of pregnancy, and considered alone, was not a reliable diagnostic indicator of the disease. Sibai, B. et al, Contemp. Ob/Gyn 57 (Feb. 1988) reports that the search continues for a reliable means for forecasting PIH and effective ways to reduce incidence. In the meantime, clinicians still continue using blood pressure criteria to guide management.

That the elevated fibronectin level observed with preeclampsia suggested endothelial injury was postulated by Bhatia, R. et al, Am. J. Obstet. Gynecol. **157**:106-108 (1987). More recently, Roberts, Ob. Gyn. News. 221 (Nov. 1987) has suggested that the evidence suggests that preeclampsia is a disease process fundamentally related to endothelial cell injury, not a hypertensive disorder.

DESCRIPTION OF THE PRIOR ART

Monoclonal antibodies which bind preferentially with human cellular fibronectin and can distinguish between the cellular fibronectin and plasma fibronectin are described by Keen, J. et al, Mol. Biol. Med. 2:15-27 (1984). Monoclonal antibodies which bind with a unique Ed sequence of cellular fibronectin in embryonic and adult human tissues are described by Vartio, T. et al, J. Cell Sci. 88:419-430 (1987). Other publications describing antibodies which distinguish certain fibronectins include publications by Atherton, B. et al, Cell 25:13-141 (1981); Carnemolla, B. et al, FEB Letters 215:269-273 (1987); Carnemolla, B. et al, J. Cell Biol. 108:1139-1148 (1989); Sekiguchi, K. et al, Biochemistry 28:3293-3298 (1989); Virtanen, I. et al, Histochemistry 90:25-30 (1988); and Zardi, L. et al, EMBO J. 6:2337-2342 (1987). Unique amino acid stretches present in human fibronectin are reported by Gutman, A. et al, Proc. Natl. Acad. Sci. USA 84:7179-7182 (1987). Peters, J. et al, Am. Rev. Respir. Dis. 138:167-174 (1988) describes the synthesis of an Extra Type III Domain (ED1) peptide corresponding to a unique nonhomologous stretch of 29 amino acids present in human cellular fibronectin, antibodies binding with this region, and an ELISA immunoassay developed with these antibodies. The peptide sequence disclosed is TYSSPEDGIHELFPAPDGEEDTAELQGGC, using the single letter abbreviations for alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), leucine (L), proline (P), glutamine (Q), serine (S), threonine (T), and tyrosine (Y).

SUMMARY OF THE INVENTION

Preeclampsia, pregnancy induced hypertension (PIH) and eclampsia are determined by identifying the presence of a fibronectin isoform binding with monoclonal antibody A134 in a sample of blood, plasma or serum of a pregnant woman using an immunoassay such as a biograting binding assay, or a sandwich or competition immunoassay.

In the biograting assay, the sample is contacted with a support having a biograting (essentially non-light disturbing diffraction grating pattern) of active A134 antibody thereon. The light diffracted by the diffraction grating formed by binding of the A134 antibody and fibronectin isoform in the sample is then determined.

In the sandwich immunoassay, the sample is contacted with an insoluble support to which A134 antibody is adhered for a time sufficient to permit antigen-antibody binding to occur. The insoluble support is then be contacted with an anti-fibronectin antibody for a time sufficient to permit antigen-antibody binding to occur; and the presence of anti-fibronectin antibody on the insoluble support is determined. The anti-fibronectin antibody can have a physically detectable label such as an enzyme label, and the level of label bound to the insoluble support following removal of unreacted secondary antibody is then determined.

One competition method of this invention comprises contacting an insoluble support to which A134 antibody is adhered with a mixture of the sample and an amount of a labeled anti-(A134 idiotype) antibody which is insufficient to bind with all of the A134 antibody adhering to the insoluble support, and determining the label adhering to the insoluble support or remaining in the mixture.

Another competition method of this invention comprises contacting an insoluble support to which anti-(A134 idiotype) antibody is adhered with a mixture of the sample and an amount of labeled A134 antibody which is insufficient to bind with all of the anti-(A134 idiotype) antibody adhering to the insoluble support, and determining the label adhering to the insoluble support or remaining in the mixture.

Another competition method of this invention comprises contacting an insoluble support to which A134 antibody is adhered with a mixture of the sample and an amount of A134 antibody which is insufficient to bind with all of the antigen in the sample. The insoluble support is subsequently reacted with labeled anti-fibronectin, and the amount of antigen adhering to the support or remaining in the mixture is determined.

Reagents of this invention comprise A134 antibody, anti-(A134 idiotype) antibody, and labeled derivatives thereof.

Testing kits of this invention comprise a combination of A134 antibody adhering to an insoluble support with other reagents required to perform the test. The other reagents can include wash solutions, labeled or unlabeled secondary binding reagents, enzyme substrates for enzymes, and the like. The reagents can be present in any suitable form in the kit, for example in separate containers, packages, and the like.

DETAILED DESCRIPTION OF THE INVENTION

This object of this invention is the detection of preeclampsia, pregnancy induced hypertension (PIH) and eclampsia by identifying the presence of A134-binding fibronectin isoform in a sample of blood, plasma or serum of a pregnant woman using an immunoassay. This isoform appears in fetal villous fibroblasts and amniotic fluid. It is distinguished from plasma fibronectin which is of hepatic origin. It appears to

be different from some cellular fibronectins since we have been unable to detect it in endothelial cell cultures. It is not present in significant amounts in maternal blood during normal pregnancy, but it may appear in maternal circulation when placental damage is present.

The term "anti-(fibronectin) antibody", as used herein, is defined to include antibodies which bind with total fibronectin components of blood, including plasma fibronectin and endothelial cell fibronectin, and antibodies which bind only with fibronectin isoforms such as cellular fibronectin.

The term "antibody", as used herein is defined to include antibodies of classes IgG, IgM, IgA, IgD, and IgE, and binding fragments, half-antibodies, and hybrid derivatives of antibodies including, but not limited to Fab, and $F(ab')_2$ fragments of antibodies.

The term "preferentially bind" and "preferentially binding", as used herein, is defined to include antibodies and fragments thereof which have less than 10 percent and preferably less than 5 percent cross-reactivity.

In the method of this invention, a blood sample is collected from the patient by conventional procedures. It can be collected from a capillary or vein. It can be drawn into an evacuated container and mixed with heparin, sodium citrate or EDTA and centrifuged to remove cells, yielding plasma. It can be clotted, and serum separated from the clot. It can be absorbed by an absorbent material such as paper and dried.

The term "immunoassay", as used herein, is defined to mean a method using a preferential binding property of A134 antibody with its A134-binding fibronectin isoform. Included within the scope of this invention are all immunoassay methods including this step, including but not limited to sandwich, competition, agglomeration, precipitation, transistor bridge probe,

particle sorting, light disturbing, light scattering, and ultrasonic probe immunoassays, for example. Appropriate immunoassays may use, as labels, radioisotopes, enzymes, or fluorogenic, chromogenic, or chemiluminescent substances.

A sandwich immunoassay embodiment of this invention for determining preeclampsia, pregnancy induced hypertension or eclampsia comprises obtaining a blood, plasma or serum sample from a pregnant patient. The sample is contacted with an insoluble support to which A134 antibody is adhered to effect binding and capture of A134-binding fibronectin isoform in the sample. The insoluble support is then contacted with an unlabeled or labeled secondary antibody which binds selectively with the bound fibronectin isoform. The captured secondary antibody is then determined. If the secondary antibody is labeled, the label can be detected or its amount measured. If the secondary antibody is unlabeled, the insoluble support can be contacted with a labeled tertiary antibody which binds preferentially with the secondary antibody, and the amount of label on the insoluble support can be determined. Alternatively, this can be accomplished by contacting a mixture of the sample and a labeled secondary antibody with an insoluble support to which the A134 antibody is adhered for a time sufficient to permit antigen-antibody binding to occur; and determining the presence or amount of the labeled secondary antibody bound to the insoluble support.

One competition immunoassay embodiment of this invention comprises contacting an insoluble support to which A134 antibody is adhered with a mixture of the sample and an amount of a labeled anti-(A134 idiotype) antibody which is insufficient to bind with all of the A134 antibody adhered to the support; and determining the amount of labeled anti-(A134 idiotype) antibody

bound to the insoluble support or remaining in the mixture.

An alternative competition immunoassay of this invention comprises contacting an insoluble support to which an anti-(A134 idiotype) antibody is adhered with a mixture of the sample and an amount of labeled A134 antibody which is insufficient to bind with all of the anti-(A134 idiotype) antibody adhered to the support; and determining the amount of labeled A134 antibody bound to the insoluble support or remaining in the mixture.

A particularly advantageous immunoassay of this invention uses a biograting. Biograting methods, materials and apparatus are described in U.S. Patents 4,647,544 and 4,876,208, the entire contents of which are hereby incorporated by reference. In this method, the sample is contacted with an A134 antibody biograting for a sufficient time to permit A134 antibody to bind with fibronectin isoform in the sample. After the sample is removed from the biograting surface, the light diffracted by the grating formed by the binding reaction is detected and measured.

The A134 antibody is produced by hybridoma cell line ATCC No. HB 10403.

The anti-(A134 idiotype) antibody can be prepared from the A134 antibody by methods described by P. Chen et al, J. Exp. Med. 162:487-500 (1985), and in PTC Application No. 8502909 and EP Application No. 141783. Monoclonal antiidiotype antibodies to A134 antibody can also be prepared by the procedures described in U.S. Patent 4,513,088.

Secondary antibodies binding with the fibronectin isoform and methods for their preparation are described by Keen, J. et al, Mol. Biol. Med. 2:15-27 (1984); Vartio, T. et al, J. Cell Sci. 88:419-430 (1987); Gutman, A. et al, Proc. Natl. Acad. Sci. USA

84:7179-7182 (1987); and Peters, J. et al, Am. Rev. Respir. Dis. 138:167-174 (1988), the entire contents of each of the above publications being hereby incorporated by reference. Other suitable secondary antibodies of both polyclonal and monoclonal varieties are generally well known and available either commercially or from publicly available hybridoma deposits. For example, anti-(total fibronectin) monoclonal antibodies can be derived from clone samples from ATCC HB91 (American Type Culture Collection, Rockville, MD). Such antibodies are also described in Japanese Patent Application 60091264 (DIALOG database file 351, WPI Acc. No. 85-161617/27) and U.S. Patent 4,325,867.

The antibody reagents can be bonded to an insoluble support by conventional processes. A variety of materials can be used as the insoluble support, the primary consideration being the binding of the A134 or anti-(A134 idiotype) antibody to the surface, the absence of interference with the reagent binding reaction or with other reactions which can be employed to determine the presence and extent of the binding reaction. Organic and inorganic polymers, both natural and synthetic, can be used as the insoluble support. Examples of suitable polymers include polyethylene, polypropylene, polybutylene, poly(4-methylbutylene), butyl rubber, silastic polymers, polyesters, polyamides, cellulose and cellulose derivatives (such as cellulose acetate, nitrocellulose and the like), acrylates, methacrylates, vinyl polymers (such as polyvinyl acetate, polyvinyl chloride, polyvinylidene chloride, polyvinyl fluoride, and the like), polystyrene and styrene graft copolymers, rayon, nylon, polyvinylbutyrate, polyformaldehyde, etc. Other materials which can be used as the insoluble support are the latexes of the above polymers, silica gel, silicon wafers, glass, paper, insoluble protein,

metals, metalloids, metal oxides, magnetic materials, semi-conductive materials, cermets and the like. In addition are included substances which form gels, e.g. proteins such as gelatins, lipopolysaccharides, silicates, agarose, polyacrylamides or polymers which form several aqueous phases such as dextrans, polyalkylene glycols (alkyene with 2 to 3 carbon atoms) or surfactants, e.g. amphophilic compounds such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts, and the like.

For membrane immunoassays, the preferred support comprises a nylon or nitrocellulose membrane. If antibody coated latex particles are used as reagents, however, the structure and composition of the membrane is not critical since the membrane functions as a mechanical filter to retrain the latex particles.

A particularly advantageous support for this procedure comprises a microtiter plate having a plurality of wells. The well surface or plastic cup inserts therein can constitute the antigen or antibody support. If the determination will require the use of fluorometric measurements, the microtiter plate or the well inserts are advantageously opaque to light so that excitation light applied to a well does not reach or influence contents of the surrounding wells. Microwells can be made from polystyrene, styrene copolymers such as styrene-acrylonitrile copolymers, or polyolefins such as polyethylene or polypropylene, and acrylate and methacrylate polymers and copolymers.

The antibodies can be bound to the insoluble support by adsorption, ionic bonding, van der Waals adsorption, electrostatic bonding, or other non-covalent bonding, or it can be bound to the insoluble support by covalent bonding. Procedures for binding antibodies to insoluble supports are described in U.S. patents 3,551,555, 3,553,310, 4,048,298 and RE-29,474, and by Tijssen (supra pp 297-328), for

example. Procedures for binding of antibodies to polystyrene by adsorption are described in U.S. patents 3,646,346 and 4,092,408, for example.

Procedures for non-covalent bonding are described in U.S. Patent 4,528,267. Procedures for covalently bonding antibodies to insoluble supports are described by Ichiro Chibata in IMMOBILIZED ENZYMES. Halsted Press: New York (1978) and A. Cuatrecasas, J. Bio. Chem. 245:3059 (1970), the entire contents of which are hereby incorporated by reference. The surface can be coated with a protein and coupled with the antibody or antigen using procedures described in U.S. Patent 4,210,418 using glutaraldehyde as a coupling agent, for example. In a still further procedure, the well can be coated with a layer having free isocyanate groups such as a polyether isocyanate, and application of the antibody or antigen in aqueous solution thereto effects the requisite bonding. In a still further procedure, the antibody or antigen can be coupled to a hydroxylated material by means of cyanogen bromide as described in U.S. Patent 3,720,760.

The insoluble supports are preferably "blocked" to reduce non-specific binding. The choice of suitable blocking agents in determined by the type of insoluble support. For example, for polystyrene supports, suitable blocking agents include water-soluble non-immune animal proteins. Suitable water-soluble non-immune animal proteins include bovine (BSA), human, rabbit, goat, sheep, and horse serum albumins; casein and non-fat milk; ovalbumin, glycoproteins, and the like.

The same blocking agents can also be used for nylon and nitrocellulose supports. However, a preferred blocking agent for nitrocellulose or nylon membrane supports is non-fat milk or casein. An optimum blocking agent for these membrane supports is an aqueous solution containing from 0.1 to 5 wt.%

non-fat dried milk or casein, and nonionic surfactants such as polyoxyethylene sorbitan derivatives and polyoxyethylene ethers.

The labeled A134 and anti-(A134 idiotype) antibody reagents of this invention can be prepared by conventional procedures for attaching labels to proteins, preferably with suitable protection of antibody binding sites. The labels can be bonded or coupled to the protein reagents by chemical or physical bonding. Ligands and groups which can be bound to the antibodies of this invention include elements, compounds or biological materials which have physical or chemical characteristics which can be used to distinguish the reagents to which they are bonded from compounds and materials in the sample being tested.

Radiolabeled antibodies of this invention can be used for in vitro diagnostic tests. The specific activity of a tagged antibody depends upon the half-life, isotopic purity of the radioactive label and how the label is incorporated into the antibody. Table A lists several commonly used isotopes, their specific activities and half-lives. In immunoassay tests, the higher the specific activity, in general, the better the sensitivity.

TABLE A

Isotope	Specific Activity of Pure Isotope (Curies/mole)	Half-Life
^{14}C	6.25×10^{1}	5720 years
^{3}H	2.91×10^{4}	12.5 years
^{35}S	1.50×10^{6}	87 days
^{125}I	2.18×10^{6}	60 days
^{32}P	3.16×10^{6}	14.3 days
^{131}I	1.62×10^{7}	8.1 days

Procedures for labeling antibodies with radioactive isotopes listed in Table A are generally known in the art. Tritium labeling procedures are described in U.S. Patent 4,302,438, for example. Iodinating, tritium labeling and ^{35}S labeling procedures especially adapted for antibodies are described by Goding, J.W. MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE. New York: Academic Press, pp 124-126 (1983) and the references cited therein. Other procedures for iodinating antibodies are described by Hunter and Greenwood, Nature 144:945 (1962), by David et al, Biochemistry 13:1014-1021 (1974), and in U.S. Patents 3,867,517 and 4,376,110. Examples of suitable systems, coupling procedures and substrate reactions therewith are disclosed in U.S. Patents Re. 31,006, B1 3,654,090, 4,214,048, 4,289,747, 4,302,438, 4,312,943, 4,376,110 and the references cited therein, for example. Examples of other suitable systems are described by Pesce et al, Clin. Chem. 20:353-359 (1974) and Wisdom, G. Clin. Chem. 22:1243 (1976).

A list of suitable enzyme classes which can be used for labeling, and specific examples for each class, are described in Table B.

TABLE B

Class	Enzyme Example
Hydrolases	Amylases
Nucleases	Polynucleotidase
Amidases	Arginase
Purine deaminases	Adenase
Peptidases	Aminopolypeptidase
Proteinases	Pepsin
Esterases	Lipases
Iron Enzymes	Catalase
Copper Enzymes	Tyrosinases
Enzymes containing Coenzymes	Alcohol dehydrogenase
Enzymes reducing cytochrome	Succinic dehydrogenase
Yellow enzymes	Diaphorase
Mutases	Glyoxalase
Desmolases	Aldolase
Oxidases	Glucose oxidase
	Horseradish peroxidase
Phosphatases	Alkaline Phosphatases
	Acid Phosphatases
Dehydrogenases	G6PDH (Glucose 6-phospho-dehydrogenase)
β-galactosidase	
Phosphorylases	
Hexokinases	

A list of suitable enzymes are described in Hawk, et al. PRACTICAL PHYSIOLOGICAL CHEMISTRY, New York: McGraw-Hill pp 306-397 (1954).

Fluorogenic and chromogenic enzymes (enzymes in the presence of which a selected substrate will produce a fluorescent or chromophore product) are useful labeling moieties. Methods for selectively conjugating enzymes to antibodies without impairing the ability of

the antibody to bind with antigen are well known in the art.

Suitable enzymes and procedures for coupling them to antibodies are described by Ichiro Chibata in IMMOBILIZED ENZYMES (supra); A. Cuatrecasas, J. Bio. Chem. (supra); Wilson, M. et al, INTERNATIONAL CONFERENCE IN IMMUNOFLUORESCENCE AND RELATED STAINING TECHNIQUES. W. Knapp et al, editors. Amsterdam: Elsevier pp 215-244 (1978); Sullivan, M. et al, Annals of Clinical Biochemistry 16:221-240 (1979); Nygren, H. et al, Medical Biology 57:187-191 (1979); Gadkari, D. et al, Journal of Virological Methods 10:215-224 (1985); Tijssen, P. et al, Analytical Biochemistry 136:451-457 (1984); Tsuruta, J. et al, The Journal of Histochemistry and Cytochemistry 33:767-777 (1985); Ishikawa, E. Journal of Immunoassay 4:209-327 (1983); and in U.S. Patent 4,190,496, for example, the entire contents of the above listed references being hereby incorporated by reference.

The preferred enzymes and suitable substrates corresponding thereto include horseradish peroxidase for which suitable substrates are o-phenylenediamine, m-phenylenediamine, o-dianisidine, and 4-chloro-α-naphthol. They also include β-galactosidase for which suitable substrates are 4-methylumbelliferyl-β-D-galactoside, p-nitrophenyl-β-D-galactose, p-nitrophenol, o-nitrophenyl-β-D-galactose, and o-nitrophenol, for example. They include alkaline phosphatase for which suitable substrates are p-nitrophenylphosphate, indoxyl phosphate, phenophthalein monophosphate, and 5-bromo-3-chloroindoxyl phosphate, for example.

Examples of suitable procedures for enzyme labeling the antibody include the use of carbodiimides, dialdehydes, and gluteraldehyde bifunctional coupling reagents. Linkage of enzymes through amine groups can be achieved by treating the proteins with thionyl chloride, N-hydroxysuccinimide or similar reagents in

an anhydrous solvent such as dimethylformamide, dioxane, dimethylsulfoxide, tetrahydrofuran, or the like. Alternative coupling agents include carbodiimides such as 1-ethyl-3-(3-(N,N'-dimethylamino)propyl)-carbodiimide, 1-cycloexyl-3-(2-morpholino-ethyl)carbodiimide, methyl-p-toluenesulfonate, succinimidyl 4-(N-maleimidoethyl)-cyclohexane-1-carboxylate, and succinimidyl 3-(2-pyridyldithio)-propionate, for example.

The carbohydrate moiety of an enzyme can also be oxidized to an aldehyde and reacted with lysyl amino groups of immunoglobulins to form a Schiff's base. Reduction with sodium borohydride effects a stable linkage of enzyme and antibody. Horseradish peroxidase with antibody can be efficiently linked to immunoglobulins by the method of M. Wilson (supra).

Fluorophore and chromophore labeled antibodies can be prepared from standard moieties known in the art. Since antibodies and other proteins absorb light having wavelengths up to about 310 nm, the fluorescent moieties should be selected to have substantial absorption at wavelengths above 310 nm and preferably above 400 nm. A variety of suitable fluorescers and chromophores are described by Stryer, Science **162**:526 (1968) and Brand, L. et al, Annual Review of Biochemistry **41**:843-868 (1972). The antibodies can be labeled with fluorophore chromophore groups by conventional procedures such as those disclosed in U.S. Patents 3,940,475, 4,289,747 and 4,376,110, for example.

One group of fluorescers having a number of the desirable properties described above are the xanthene dyes, which include the fluoresceins derived from 3,6-dihydroxy-9-phenylxanthhydrol and resamines and rhodamines derived from 3,6-diamino-9-phenylxanthydrol and lissanime rhodamine B. The rhodamine and fluorescein derivatives of 9-o-carboxyphenylxanthhydrol

have a 9-o-carboxyphenyl group. Fluorescein compounds having reactive coupling groups such as amino and isothiocyanate groups such as fluorescein isothiocyanate and fluorescamine are readily available. Another group of fluorescent compounds are the naphthylamines, having an amino group in the α or β position.

Antibodies can be labeled with fluorochromes or chromophores by the procedures described by Goding, J.W. (supra, pp 208-249).

The antibodies used in the methods of this invention can be covalently bonded to avidin or biotin in one embodiment of this invention. Suitable binding procedures involve cross-linking through a bifunctional cross-linking agent. Suitable bifunctional compounds are described by Peters, K. et al, Ann. Rev. Biochim. **46**:523 (1977).

In other instances, the bonds can be formed directly between the reagents themselves. For example, antibody can be bound to avidin through functional groups on the respective materials. As a specific example, avidin can be treated with periodate and reacted with antibody to give a Schiff base formation without inhibiting the biotin to avidin binding or blocking immunological activity of the antibody.

Known techniques using bifunctional cross-linking agents include the following: (a) a one-step glutaraldehyde linkage, Avrameas, S. Immunochemistry **6**:43 (1969); (b) two-step glutaraldehyde linkage, Avrameas, S. Immunochemistry **8**:1175 (1971); and (c) dimaleimide linkage, Kato, K. et al, Euro. J. Biochem. **62**:285 (1966).

Antibodies can be labeled with metallic radionuclides according the procedure of Hnatowich, D. et al. Journal of Applied Radiation 35:554-557 (1984) and Buckley, R et al. Federation of European Biochemical Societies **166**:202-204 (Jan. 1984).

One embodiment of the immunoassay methods of this invention uses an insoluble support such as a polystyrene plate to which A134 antibody or anti-(A134 idiotype) antibody is adhered, either directly or through a goat anti-mouse antibody. It is contacted with a sample diluted with an aqueous buffer solution such as phosphate buffer solution (PBS), pH 6 to 8 and preferably from 7.2 to 7.6 for a sufficient time to permit binding of antibody in the sample with the antibody on the insoluble support, and then removing the sample from the support. The incubation time should be sufficient to permit substantial binding to occur, the time being temperature dependent. Suitable incubation times are from 30 to 240 minutes at temperatures within the range of from 16 to 40°C, the preferred contact time being at least 30 minutes at temperatures within the range of from 20 to 26°C. The residual sample solution is then removed from the support by use of a rinse solution. Any conventional rinse solution can be used. Preferred buffers are tris (tromethamine) buffered solutions. A phosphate buffered rinse solution such as is described in U.S. Patent 4,528,267 can also be used if it is contains no free phosphate. It is an aqueous phosphate buffer solution having a phosphate molarity of from 0.0001 to 0.05, a pH of from 6 to 8 and containing from 0.001 to 0.1 weight percent of non-ionic surfactant. Suitable non-ionic surfactants include polyoxyethylene ethers (BRIJ such as lauryl, cetyl, oleyl, stearyl, and tridecyl polyoxyethylene ethers); polyoxyethylene sorbitans (TWEEN such as polyoxyethylene sorbital monolaurate, monopalmitate, monostearate, monoleate, and trioleates); and other polyoxyethylene ethers (TRITON, for example). Any free phosphate present in residual rinse solution remaining on the insoluble support will react during the substrate development and reduce the sensitivity of the method.

For preparation of biogratings, any smooth surface having the requisite high binding affinity for binding reagent can be used in this process. For purposes of clear explanation and not by way of limitation, the process is described for a semiconductor wafer with a polished surface bearing a polysilicon coating. It should be understood that the same, equivalent, or similar procedures can be applied for preparing diffraction gratings designs with binding reagents with other high binding smooth surfaces. With the preferred insoluble supports of aluminum, silicon nitride, silicon dioxide, single crystalline silicon, and particular the polysilicon surfaces, the binding reagent can be applied by simple adsorption. In one procedure for non-covalent adhesion of binding reagent to the surface of an insoluble support, the antibody is applied to the surface of the support by immersing the surface in a buffered binding reagent solution for from 0.5 to 18 hours and preferable from 1 to 3 hours, at temperatures of from 4 to 40°C and preferable from 20 to 26°C. The polysilicon surface is then rinsed with a buffered saline solution and dried.

The concentration of binding reagent in the buffer solution is selected to provide the desired reagent density on the polysilicon surface. The binding reagent solution can contain from 0.02 to 100 μgrams/ml of the binding reagent and preferably contains from 10 to 50 μgrams/ml of the binding reagent in a buffered solution having a pH of from 6.0 to 9.5 and preferably from 7.0 to 8.5.

A suitable rinse solution is an aqueous phosphate buffer solution such as is described in U.S.Patent 4,528,267 having a phosphate molarity of from 0.0001 to 0.05, a pH of from 6 to 8 and containing from 0.001 to 0.1 weight percent non-ionic surfactant and from 0.0001 to 0.5 weight percent of an animal serum albumin. Suitable non-ionic surfactants include polyoxyethylene

ethers (BRIJ) such as lauryl, cetyl, oleyl, stearyl, and tridecyl polyoxyethylene ethers; polyoxyethylene-sorbitans (TWEEN) such as polyoxyethylenesorbitan monolaurate, monopalmitate, monostearate, monoleate and trioleates; and other polyoxyethylene ethers (TRITON), for example. Preferred non-ionic surfactant are the polyoxyethylenesorbitans such as polyoxyethylenesorbitan monolaurate (TWEEN 20).

A mask is prepared by photographic methods conventional in semiconductor manufacturing. For example, a mask having a plurality of diffraction gratings with the desired line density and line widths can be prepared on a quartz glass or other UV-transparent plate through a photoresist process similar to photography. The dark lines of the mask correspond to active binding reagent areas desired on the ultimate surface.

The mask is placed over the polysilicon surface having a coating of binding reagent, and the surface is exposed to ultraviolet radiation until the binding capability of the portions of the binding reagent exposed to the radiation is substantially reduced or preferably eliminated. To manufacture a precision grating design, the radiation should form a sharp image on the coated surface. Penumbrae should be minimized. Preferably, the ultraviolet light passing through the mask is focused to a sharp image on the surface coating using conventional projection alignment techniques without contact with the coated surface.

The ultraviolet radiation exposure required to deactivate antibody coating exposed thereto is from 30 sec to 60 min and preferably from 45 to 60 min with ultraviolet radiation having a wavelength such as 254 nm and a power of from 8 to 14 mwatts per cm^2. Some adjustment in time of exposure and/or power may be necessary to deactivate the binding sites of other antibody binding agents.

This treatment reduces or eliminates the binding properties of the binding reagent in exposed areas, leaving active binding reagent in a diffraction grating design corresponding to the opaque areas of the mask. The coated substrate containing areas having binding protein in a diffraction grating design is cut into smaller area chips, each chip having a size sufficient to perform an binding assay. These chips are then mounted on a suitable diagnostic support such as the dipstick.

The first step in the biograting binding assay method according to this invention comprises contacting a diffraction binding assay surface with the sample of blood, plasma, or serum of a pregnant woman, for a time sufficient to permit conjugation of active zones of A134 antibody with A134 binding fibronectin isoform in the sample. The diffraction binding assay surface is an insoluble surface with a light disturbing design of substantially non-light disturbing A134 thereon.

The surface is then separated from the sample. The surface is preferably rinsed, for example with distilled or deionized water, and the excess water is removed.

The insoluble surface is then illuminated with light from a light source, and the diffraction of light by the insoluble surface is determined. The concentration of the A134 binding fibronectin isoform present correlates with the diffraction intensity determined.

The strength of the light diffraction is measured with a suitable light diffraction instrument such as is illustrated hereinabove, adjusted to provide the angle of incidence, α, within suitable ranges. The relative strengths of the light diffracted is a function of the amount of primary binding reagent-analyte conjugate comprising the grating. By repeating the above procedure with a prepared series of solutions

containing a range of different known concentrations of analyte therein, a standard curve functionally related to the strength of the diffracted light is obtained. By comparing the reading obtained with the sample containing the analyte with the curve obtained with solutions containing known concentrations of the analyte, the concentration of analyte in the sample can be determined. Comparing the strengths of the first, second, third, etc. order diffractions with each other and with the strength of the reflected light directly provides an indication of the degree of conjugation with the grating binding agent.

In sandwich and enzyme labeled immunoassays, further treatment of the support carrying the binding reaction product of the A134 antibody and the sample fibronectin isoform is required to determine the extend of the binding, that is, the relative amount of the fibronectin isoform on the surface.

In sandwich immunoassays, the insoluble support having the A134-fibronectin isoform product thereon is then contacted with a secondary or sandwiching antibody which will bind with the captured fibronectin on the insoluble support. The secondary antibody can be labeled or unlabeled. In the event that an unlabeled secondary antibody is used, a tertiary antibody which binds with the secondary antibody and which bears a physically detectable label can be used in a conventional manner to determine the sandwiching antibody.

A variety of labels have been described above. For purposes of clarity and not by way of limitation, the subsequent steps of the process will be described for anti-fibronectin antibodies which have been labeled with an enzyme, preferably a chromogenic or a fluorogenic enzyme. The term "chromogenic enzyme" is defined herein to refer to an enzyme which will produce a chromophore product with a suitable substrate. The

term "fluorogenic enzyme" is defined herein to refer to an enzyme which will produce a fluorophore product with a suitable substrate.

The secondary antibody is applied to the insoluble support in an aqueous solution. The solution preferably contains suitable salts and buffers to preserve the reactants and facilitate the binding reaction. For example, the solution can contain bovine serum albumin (BSA), phosphate buffer solution (PBS), and a mild surfactant such as polyoxyethylene sorbitan ester employed in the above-described rinse solution. The incubation is continued for sufficient time to permit the secondary antibody to bind with exposed epitopes of A134 binding fibronectin isoform, if any, adhering to the insoluble support.

The secondary antibody solution is then removed from the insoluble support, and the support is rinsed with a rinse solution such as described above, to remove any residual, unbound labeled material.

If the secondary antibody is unlabeled, an enzyme labeled antibody or other binding agent which binds selectively with the secondary antibody is applied to the insoluble support in an aqueous solution. The solution preferably contains suitable salts and buffers to preserve the reactants and facilitate the binding reaction. For example, the solution can contain bovine serum albumin (BSA), phosphate buffer solution (PBS), and a mild surfactant such as polyoxyethylene sorbitan ester employed in the above-described rinse solution. The incubation is continued for time to permitting labeled tertiary antibody to bind with epitopes of secondary antibody, if any, adhering to the insoluble support.

The labeled antibody solution is then removed from the insoluble support, and the support is rinsed with a rinse solution such as described above, to remove any residual, unbound labeled material.

In a next step of the sandwich process of this invention, the insoluble support is contacted with an aqueous solution of a substrate which undergoes a reaction in the presence of the enzyme to release a fluorophore or chromophore into the solution. Suitable substrates and the enzymes which they can be converted are described in U.S. Patents 4,190,496 and 4,528,267, for example. The support is contacted with an aqueous solution of the substrate containing from 10^{-2} to 10^{-10} molar concentrations of the substrate. Substrate molar concentrations of from 10^{-4} to 10^{-5} are preferred. Preferred additional reagents and buffers in the substrate solution include 2-amino-2-methyl-1-propanol buffer, TRIS, and magnesium chloride, for example.

The substrate solution is incubated with the insoluble support for sufficient time for the reaction yielding the fluorophore or chromophore to occur. At temperatures of from 18 to 40°C, incubation times of from 5 to 240 minutes can be used. Preferably, the temperature is within the range of from 20 to 26°C, and the incubation time is from 30 to 120 minutes.

The fluorophore or chromophore level in the solution is then measured. The equipment and procedures for determining the level of fluorescence or chromophore level in the substrate solutions are those conventionally used in the art. The level of fluorescence or chromogen in solution is a function of the enzyme concentration on the insoluble support which is, in turn, a function of the amount of A134-binding fibronectin isoform in the sample. The concentration of the fibronectin isoform can be determined by comparing the fluorescence or chromophore level of the solution with control solutions containing known concentrations of A-134 reactive cellular fibronectin. The presence of the A134 binding fibronectin isoform indicate the presence of preeclampsia, eclampsia or PIH condition.

In a membrane embodiment of the immunoassay methods of this invention, a membrane support to which A134 antibody is adhered is contacted with blood, serum or plasma sample diluted with an aqueous buffer solution such as phosphate buffer solution (PBS), pH 6 to 8 and preferably from 7.2 to 7.6 for a sufficient time to permit binding of A134 binding fibronectin isoform in the sample with the A134 antibody on the insoluble support. The time required for binding is very small in a flow through system. Suitable incubation times can be one sec up to 20 min at temperatures within the range of from 16 to 40°C, the preferred contact time being less than one min and optimally from 10 sec to 2 min.

The insoluble support is then contacted with an anti-fibronectin antibody, the secondary antibody. The secondary antibody can be labeled or unlabeled. In the event that an unlabeled sandwiching antibody is used, a secondary antibody which binds with the sandwiching antibody and which bears a physically detectable label can be used in a conventional manner to determine the sandwiching antibody.

A variety of useful labels have been described above. For purposes of clarity and not by way of limitation, the subsequent steps of the process will be described for anti-fibronectin antibodies which have been labeled with a chromogenic enzyme.

The secondary antibody is applied to the insoluble support in an aqueous solution. The solution preferably contains suitable salts and buffers to preserve the reactants and facilitate the binding reaction. For example, the solution can contain bovine serum albumin (BSA), phosphate buffer solution (PBS), and a mild surfactant such as polyoxyethylene sorbitan ester employed in the above-described rinse solution. The incubation is continued for sufficient time to permit the sandwiching antibody to bind with exposed

fibronectin epitopes, if any, adhering to the insoluble support.

The secondary antibody solution optionally can be removed from the insoluble support, and the support is rinsed with a rinse solution such as described above, to remove any residual, unbound labeled material.

If the secondary antibody is unlabeled, an enzyme labeled antibody or other binding agent which binds selectively with the secondary antibody is applied to the insoluble support in an aqueous solution. The solution preferably contains suitable salts and buffers to preserve the reactants and facilitate the binding reaction. For example, the solution can contain bovine serum albumin (BSA), phosphate buffer solution (PBS), and a mild surfactant such as polyoxyethylene sorbitan ester employed in the above-described rinse solution. The incubation is continued for sufficient time to permit labeled anti-(cellular fibronectin) antibody to bind with exposed cellular fibronectin epitopes, if any, adhering to the insoluble support. The preferred incubation times and temperatures are as set forth for the binding of insolubilized reagent anti-fibronectin antibody with the endocellular fibronectin antigen (or complex thereof).

The labeled antibody solution is then removed from the insoluble support, and the support is rinsed with a rinse solution such as described above, to remove any residual, unbound labeled material.

In a next step of the sandwich method of this invention, the insoluble support is contacted with an aqueous solution of a substrate which undergoes a reaction in the presence of the enzyme to release chromophore compound into the solution. Suitable substrates and the enzymes which they can be converted are described in U.S. Patents 4,190,496 and 4,528,267, for example. The support is contacted with an aqueous solution of the substrate containing from 10^{-2} to 10^{-10}

molar concentrations of the substrate. Preferred additional reagents and buffers in the substrate solution include 2-amino-2-methyl-1-propanol buffer, TRIS, and magnesium chloride, for example.

The substrate solution is incubated with the insoluble support for sufficient time for the reaction yielding the fluorophore or chromophore to occur. At temperatures of from 18 to 40°C, incubation times of from 1 to 20 min can be used. Preferably, the temperature is within the range of from 20 to 26°C, and the incubation time is from 2 to 5 min. The chromogen level on the membrane can be measured using a reflectometer or densitometer.

The kits of this invention comprise combinations of buffers for transport and storage with sampling devices such as sampling devices; supports having reagents of this invention adhered thereto; vials, foil packages or other containers of reagents of this invention; and combinations thereof. Each of the insoluble support structures in a foil package can be combined with other reagents in vials or other packages. They can also be combined with other, optional reagents such as stop reagents in separate vials or other packages.

Reagents of this invention include A134 antibody, anti-(A134 idiotype) antibody, and labeled derivatives thereof; or A134 antibody or anti-(A134 idiotype) antibody adhered to an insoluble reagent support.

The testing kits of this invention are combinations of reagents which are used together in the respective tests. The kits include but are not limited to the following combinations:

a) A134 antibody adhered to an insoluble support and a labeled anti-fibronectin secondary antibody;

b) A134 antibody adhered to an insoluble support and labeled anti-(A134 idiotype) antibody;

c) Anti-(134 idiotype) antibody adhered to an insoluble support and labeled A134 antibody; and

d) A134 antibody biograting and reference biograting.

This invention is further illustrated by the following specific, but non-limiting examples. Temperatures are given in degrees Centigrade and percents as weight percents unless otherwise specified. Examples which are constructively reduced to practice herein are presented in the present tense, and examples representing laboratory experiments previously reduced to practice are presented in the past tense.

EXAMPLE 1

Polyclonal Anti-fibronectin Antibodies

Cellular fibronectin is purified from human fetal villous fibroblast cells as described by Laine et al, Meth. Enzymol. **144**:420-429.

The anti-(cellular fibronectin) antibodies are elicited in rabbits using the immunization techniques and schedules described in the literature, e.g. Herbert, HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, App. 2 and 3, Ed. D.M. Weir (1973), immunizing the rabbits with the cellular fibronectin antigen. The antiserum titer is determined by the level of its binding to cellular fibronectin in a solid phase assay similar to that used for monoclonal antibodies, e.g., as described by Lange, et al Clin. Exp. Immunol. **25**:191 (1976) and Pisetsky, et al J. Immun. Meth. **41**:187 (1981).

The IgG fraction of the antisera is purified further by affinity chromatography using Protein G Fast Flow (Pharmacia) using the manufacturer's instructions, followed by affinity chromatography using CNBr-Sepharose 4B (Pharmacia) to which has been coupled fetal villous fibroblast cellular fibronectin. The method used for coupling is that recommended by the gel

manufacturer (AFFINITY CHROMATOGRAPHY, pp. 15-18, Pharmacia Fine Chemicals).

Finally, the affinity purified IgG is absorbed with soluble plasma fibronectin to remove any antibodies which would cross-react with plasma fibronectin. The absorbed antibody-plasma fibronectin complexes are then removed by further chromatography through an anti-(plasma fibronectin) column, which will bind the complexes and allow the pure, anti-fetal villous fibroblast fibronectin to pass through.

EXAMPLE 2

A134 Monoclonal Antibody

Using a membrane preparation derived from fetal villous fibroblasts, mouse monoclonal antibodies to fibronectin isoforms indicative of PIH, eclampsia and preeclampsia were obtained using standard procedures of Oi and Herzenberg, SELECTED METHODS IN CELLULAR IMMUNOLOGY, pp 351-371, Ed. Mishell and Shiigi (1980). Balb-c mice were immunized with membranes prepared by the technique of Mehdi and Nussey, Biochem. J. (1975) from fibroblasts derived from the primary culture of fetal villous fibroblasts. The immunized mouse spleen cells were fused with SP/2 mouse myeloma fusion partners. The fused cells were plated by limiting dilution to produce largely monoclonal colonies. The hybrids growing in HAT medium were selected and screened for production of antibodies binding preferentially with fibroblasts of fetal origin, as opposed to fibroblasts of maternal or adult origin, using fluorescence immunohistology on fibroblasts growing in vitro. The clones were further selected based on their binding to fibronectin coated polystyrene microtiter plates using ELISA methods.

The A134 hydridoma product, denoted A134 antibody, was found to bind preferentially to tissue derived fibronectin and amniotic fluid-derived fibronectin,

rather than plasma fibronectin, if the antibody was immobilized and the fibronectin was soluble and unbound. The clone was subcloned to insure monoclonality.

The A134 hybridoma antibody was purified from hybridoma culture supernatant using Protein G Sepharose affinity chromatography (Pharmacia). A134 hybridoma antibody from ascitic fluid was purified using Protein G Sepharose affinity chromatography, and further purified using an A134 fibronectin affinity column.

Further studies were conducted with the A134 antibody to determine its binding characteristics. It was found to recognize an isoform of human fibronectin because:

(a) it preferentially binds to fetal fibroblasts and not with maternal fibroblasts (in vitro immunofluorescence);

(b) it preferentially binds to insoluble (immobilized on polystyrene) plasma fibronectin and not to soluble plasma fibronectin; and

(c) it preferentially binds with soluble amniotic fluid fibronectin and soluble cellular fibronectin but not with soluble plasma fibronectin.

Investigations of epitope binding sites indicated that the epitope binding site is most likely within a 60-70 kd region encompassing fibronectin type III domains III-4 through III-7.

The A134 antibody is an IgG1 type monoclonal antibody, produced by the A134 hybridoma ATCC deposit no. HB 10403.

EXAMPLE 3

A134 Antibody Coated Microtiter Plate

A134 antibody produced as described in Example 2 was diluted to 10 μg/mL in 0.01 M PBS pH 7.5. 100 μL were dispersed into each well of a polystyrene

microtiter plate (Costar). The plate was covered and incubated 4 hr at room temperature or 4°C overnight. The plate was washed 4 times with Wash Buffer (0.02 M Tris HCl, 0.015 M NaCl, 0.05% TWEEN-20), filling and emptying the wells completely with each use. The plate was then blocked and stabilized by dispensing into each well 200 µL of a blocking/stabilizing solution (0.01 M PBS, 4 % sucrose, 1% mannitol, 1% BSA, 0.02% NaN_3, pH 7.4) and incubating for 1 hr at room temperature. The wells were then aspirated to dryness and stored sealed with a desiccant pack. The plate was then ready for immunoassay of samples.

EXAMPLE 4

Enzyme Labeled Anti-fibronectin Antibody

Anti-fibronectin antibody prepared in accordance with the procedures of Example 1 or Example 2 is conjugated with alkaline phosphatase following the one-step glutaraldehyde procedure of Avrameas, Immunochemistry. 6:43 (1969).

EXAMPLE 5

Sandwich Immunoassay

Calibrators were included in the test. The positive calibrators were A134-affinity purified human cellular fibronectin of known concentration, appropriately diluted to fall within the assay range (20 ng/mL to 5 µg/mL). The negative calibrator was sample diluent.

The sample diluent was the anti-protease cocktail described in copending, commonly assigned, U.S. patent application Serial No. 07/244,984 filed Sept. 15, 1988, the entire contents of which are hereby incorporated by reference. It protects fibronectin-containing samples from proteolytic degradation during transit and storage. The solution consisted of 0.05 M Tris-HCl, pH 7.4; 0.15 M NaCl, 0.02% NaN_3, 1% BSA, 500 Kallikrein

Units/mL of aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM EDTA.

Blood samples were obtained from two patient populations, those with and those without preeclampsia. The samples were collected and processed to yield plasma.

A microtiter plate prepared as in Example 3 was used for the assay. 100 µL of each calibrator and sample, were placed in separate, duplicate wells and incubated for 2 hr at room temperature. The plate was washed 4 times with Wash Buffer as described in Examples 3. 100 µL of alkaline phosphatase-conjugated goat anti-(human fibronectin) prepared as in Example 4 was diluted 1/100 in Conjugate Buffer (0.02 M Tris-HCl, pH 8, 0.3 M NaCl, 0.05% TWEEN 20, 5% BSA, 0.02% NaN_3). 100 µL was dispensed into each well and incubated for 2 hr at room temperature. The plate was washed 4 times as previously described. 4 mg/mL of p-nitrophenyl-phosphate (PNPP) was used as the substrate. This was diluted in 0.18 M 2-amino-2-methyl-1-propanol (AMP) buffer, pH 9.5 with 0.12 mM $MgCl_2$. 100 µL was dispensed into each well of the microtiter plate. After a 5 to 10 min incubation at room temperature, the kinetic reaction rate in milli-OD/min was read at 405 nm on a V-MAX~ kinetic microtiter plate reader (Molecular Devices).

A standard curve was constructed by correlating increasing reaction rate with increasing cellular fibronectin concentration in the calibrators. A standard curve was constructed by correlating increasing reaction rate with increasing cellular fibronectin concentration in the standards. Unknowns were calculated directly from the curve or by using a pre-set computer program (Molecular Devices). The value obtained for each plasma sample was correlated with the presence or absence of preeclampsia, and the results are shown in Table C.

TABLE C

Preeclamptic	Control
6.24	2.30
9.84	4.61
15.10	4.57
8.53	3.32
8.78	4.67
13.53	3.30
12.36	11.34
7.84	3.27
7.62	9.08
13.63	3.20
21.04	2.50
10.63	2.83
12.35	1,50
3.20	1.60
1.40	3.10
4.40	1.00
11.15	1.60
14.34	7.53
9.74	3.74
20.75	4.87
7.51	3.52
7.75	5.14
7.32	3.54
10.33	6.40

TABLE C, continued

	Preeclamptic		Control
N=	24		24
$\bar{X}$=	10.22		4.11
S=	4.65		2.39
Σx=	245.38		98.53
Σx^2	3026.66		542.07
$(\Sigma x)^2$	60211.34		9708.16
$\Sigma x^2-(\Sigma x)^2/n$=	517.86		137.56
df=		23	
pooled s^2=		14.25	
t=		5.147	
p=		± 0.001	

The preeclamptic patient group is significantly different from the nonpreeclamptic group at p= ± 0.001.

EXAMPLE 6

Anti-(A134 idiotype) Monoclonal Antibodies

Using the purified A134 antibody, polyclonal antiidiotype antibodies to the A134 antibody are obtained by immunizing goats using standard procedures of Herbert, HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, App. 2 and 3, Ed. Weir, 1973) using A134 antibody as the antigen for immunizing the goats. The goat antiserum is screened for binding to A134 antibody, and subsequently affinity purified using an A134 affinity column. The resultant goat antibody is further absorbed with nonspecific mouse, human, and rabbit IgG to eliminate cross reactivity with non-A134 antibodies.

EXAMPLE 7

A134 Antibody Diffraction

Biograting Preparation

An aminopropyl triethoxysilane activated silicon wafer is immersed in a solution of A134 antibody diluted in 0.01 M phosphate buffered solution, pH 7.5, with 0.1 wt.% sodium azide as preservative for 2 hrs at room temperature or overnight, refrigerated. The wafer is then removed from the antibody solution, rinsed with 0.05 M Tris buffer pH 8.5 containing 5 wt% sucrose and 0.5 wt% human serum albumin, and dried.

A photomask with diffraction grating lines having a line width, w, of 10 μm, and a line spacing, d, of 10 μm is placed in a Karl Suss Model 40 Mask Aligner UV exposure device (Karl Suss, Waterbury Center, Vermont 05677), and exposed to ultraviolet light having a wavelength of 254 nm and a strength of 9.6 milliwatts per cm^2 for 2 min.

The wafer is then cut into rectangular chips having a diffraction grating pattern of A134 antibody thereon, and mounted on the surface of a polyethylene dipstick.

EXAMPLE 8

Biograting Immunoassay

Patient serum from a pregnant patient is added to the surface of a polysilicon chip product of Example 7 and incubated for one hr. The serum is removed, and the chip is rinsed thoroughly with distilled water, and excess water is removed. The diffraction grating pattern of active A134 antibody binds with anti-(A134 binding fibronectin isoform) in the serum.

The intensity of the light diffracted by the grating is then measured using monochromatic light having a wavelength of 632.8 nm from a helium-neon laser.

The procedure is repeated with a positive control containing a known amount of cellular fibronectin to establish a reference point cut off with which the intensity of the diffracted light obtained with the patient serum sample is compared to determine the presence or absence of a preeclampsia, eclampsia or PIH condition.

WHAT IS CLAIMED IS:

1. A method for determining preeclampsia, pregnancy induced hypertension or eclampsia comprising
   a) obtaining a blood, plasma or serum sample from a pregnant patient;
   b) determining the presence of A134-binding fibronectin isoform in the sample.

2. A method of Claim 1 wherein the presence of the fibronectin isoform in the sample is determined by
   a) contracting the sample with an A134 antibody, and
   b) determining the binding of the antibody with the A134 binding fibronectin isoform.

3. A method of Claim 2 comprising the steps of
   a) contacting the sample with an insoluble support to which A134 antibody is adhered for a time sufficient to permit A134 binding with fibronectin isoform to occur;
   b) contacting the insoluble support with a secondary anti-fibronectin antibody for a time sufficient to permit antigen-antibody binding to occur; and
   c) determining the presence of secondary anti-fibronectin antibody on the insoluble support.

4. A method of Claim 3 wherein the secondary anti-fibronectin antibody has a physically detectable label.

5. A method of Claim 2 comprising the steps of
   a) contacting a mixture of the sample and an labeled anti-(fibronectin) antibody with an insoluble support to which A134 antibody is adhered for a time sufficient to permit antigen-antibody binding to occur; and
   b) determining the presence of labeled anti-(fibronectin) antibody on the insoluble support.

6. A method of Claim 2 comprising
   a) contacting an insoluble support to which A134 antibody is adhered with a mixture of the sample and an amount of a labeled anti-(A134 idiotype) antibody which is insufficient to bind with all of the A134 antibody adhered to the support; and
   b) determining the amount of labeled anti-(A134 idiotype) antibody bound to the insoluble support or the amount of labeled anti-(A134 idiotype) antibody remaining in the mixture.

7. A method of Claim 2 comprising
   a) contacting an insoluble support to which an anti-(A134 idiotype) antibody is adhered with a mixture of the sample and an amount of a labeled A134 antibody which is insufficient to bind with all of the anti-(A134 idiotype) antibody adhered to the support; and
   b) determining the amount of labeled A134 antibody bound to the insoluble support or the amount of labeled A134 antibody remaining in the mixture.

8. A method of Claim 2 comprising,
   a) contacting a biograting with a sample from a pregnant patient to determine the presence or quantity of A134 binding fibronectin isoform in the sample, the binding assay biograting comprising a substantially flat substrate having a uniform coating thereon, the uniform coating comprising a diffraction grating pattern of alternating zones of active and deactivated A134 antibody;
   b) illuminating the biograting with light from an optical radiation source; and
   c) detecting light diffracted by the biograting with a light detector to obtain a signal correlating with the presence or quantity of the fibronectin isoform in the sample.

9. A method of Claim 8 wherein the wherein the zones of active and deactivated A134 antibody are linear zones.

10. An A134 antibody.

11. An antibody of Claim 10 conjugated to a label.

12. An antibody of Claim 10 bound to an insoluble support.

13. A biograting comprising alternating active and deactivated zones of the antibody of Claim 10.

14. An anti-(A134 idiotype) antibody.

15. An antibody of Claim 14 conjugated to a label.

16. An immunoassay kit for detecting enclampsia, preeclampsia or PIH in pregnant women comprising
   a) an antibody of Claim 10 adhered to an insoluble support, and a labeled anti-fibronectin antibody or a labeled anti-(A134 idiotype) antibody; or
   b) an anti-(A134 idiotype) antibody adhered to an insoluble support, and a labeled A134 antibody.

AMENDED CLAIMS

[received by the International Bureau on 17 September 1991 (17.09.91); original claims 1-9 cancelled; original claim 16 added; other claims unchanged (1 page)]

10. An A134 antibody.

11. An antibody of Claim 10 conjugated to a label.

12. An antibody of Claim 10 bound to an insoluble support.

13. A biograting comprising alternating active and deactivated zones of the antibody of Claim 10.

14. An anti-(A134 idiotype) antibody.

15. An antibody of Claim 14 conjugated to a label.

16. An immunoassay kit for detecting <u>eclampsia</u> [enclampsia], preeclampsia or PIH in pregnant women comprising

a) an antibody of Claim 10 adhered to an insoluble support, and a labeled anti-fibronectin antibody or a labeled anti-(A134 idiotype) antibody; or

b) an anti-(A134 idiotype) antibody adhered to an insoluble support, and a labeled A134 antibody.

17. A hybridoma secreting an A134 antibody.

18. Hybridoma HB10403.

STATEMENT UNDER ARTICLE 19

New claims 17 and 18 are directed toward hybridomas that secrete A134 antibodies and the deposited hybridoma, Hybridoma HB10403, which produces A134 antibodies. The originally filed claims failed to claim the source of the antibodies.

Claims 1-9 have been canceled in light of the cited references. The claimed subject matter of the application is properly directed toward A134 antibodies, their sources, related antibodies and their use in detecting pregnancy induced hypertension and eclampsia. The application is now so limited. It is believed that the amendments do not require changes to the Specification. However, the Title and Abstract may be amended to more clearly reflect the scope of the claimed subject matter.

The amendments more clearly recite Applicant's invention and more clearly distinguish over the cited references.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/0222(

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: G 01 N 33/68,G 01 N 33/577,G 01 N 33/541,G 01 N 33/535, C 07 K 15/28

II. FIELDS SEARCHED

Minimum Documentation Searched †

Classification System	Classification Symbols
IPC[5]	G 01 N 33/00,C 07 K 15/00,C 12 P 21/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ‡

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ‡‡ with indication, where appropriate, of the relevant passages ‡‡	Relevant to Claim No. ‡‡
X	EP, A2, 0 359 274 (ASPEN DIAGNOSTICS CORPORATION) 21 March 1990 (21.03.90), see totality. --	1-9,16
A	EP, A2, 0 316 919 (ASPEN DIAGNOSTICS INC.) 24 May 1989 (24.05.89), see examples 2,5,7. --	1-9, 11-16
A	DE, A1, 3 743 402 (TAKARA SHUZO CO., LTD.) 07 July 1988 (07.07.88), see abstract. --	10
P,A	EP, A2, 0 399 271 (LOCUS GENEX OY) 28 November 1990 (28.11.90), see totality, esp. claim 8. --	1-9, 11,12 16
A	EP, A1, 0 048 357 (LA JOLLA CANCER RESEARCH	1-12

* Special categories of cited documents: ††

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 July 1991	22. 07. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	FOUNDATION) 31 March 1982 (31.03.82), see example 3 and the abstract. --	
P,A	Patent Abstracts of Japan, unexamined applications, C section, Volume 14, No. 265, June 08, 1990 (08.06.90), The Patent Office Japanese Government, page 30 C 726, see the abstract-No. 2-76 598(A). --	10
A	Febs Letters, Volume 215, No. 2, May 1987, B. Carnemolla et al. "Location of the cellularfibronectin-specific epitope recognized by the monoclonal antibody IST-9 using fusion proteins expressed in. E. coli", pages 269-273, see abstract. ----	10

ANHANG
zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX
to the International Search Report to the International Patent Application No.

ANNEXE
au rapport de recherche international relatif à la demande de brevet international n°

PCT/US 91/02220 SAE 46572

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsibilité de l'Office.

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